Project Title: Postharvest control of Botrytis on cut roses

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Summary

We assessed the degree to which 17 synthetic and non-synthetic fungicides controlled postharvest development of disease caused by *Botrytis cinerea* in 'Light Orlando' cut roses. Fungicides varied greatly in protection against botrytis once flowers were placed in vases at room temperature after they were inoculated with *B*. *cinerea* spores before cold storage. Phytotoxicity response of flowers also varied greatly among the fungicide treatments. Roses treated with the non-synthetic essential oil (cinnamon or thyme) fungicides at 0.20% exhibited relatively low termination frequency due to botrytis, but also exhibited moderate phytotoxicity response to the treatments. Only roses treated with the synthetic fungicide fludioxonil at 0.23 g/L had the desirable combination of low termination frequency due to botrytis and low phytotoxicity response.

Report

The objective of this research was to evaluate 17 fungicide treatments, including commercial synthetic products and non-synthetic products, for their control of postharvest development of disease caused by *B*. *cinerea* in cut roses. We focused on fungicide application before storage because effective control of botrytis during production is not always achieved and shipping and storage conditions are often conducive to *B*. *cinerea* growth. Testing synthetic and non-synthetic products may reveal new effective methods for botrytis control, important because of *B*. *cinerea* resistance to some synthetic fungicides (Hahn 2014) and the growing preference of producers, retailers, and consumers for natural, biodegradable alternatives for disease control (Wisniewski et al., 2001).

Materials and Methods

Production of rose-specific Botrytis cinerea *spores*. To ensure that we were using a *B*. *cinerea* strain that infects roses, we cultured a 25 mm^2 piece of rose petal with sporulating *B*. *cinerea* on sterile, potato dextrose agar culture medium. A 9 mm^2 plug of culture medium with vegetative mycelium from the rapidly growing forward edge of the initial culture was subcultured to a fresh plate of the same medium after 4 d, and that process was repeated after another 4 d to obtain a *B*. *cinerea* strain free of contaminants. These and all subsequent vegetative *B*. *cinerea* culture plates were sealed with parafilm and placed into an incubator held at constant 20 °C with 16 h daylight provided by fluorescent bulbs.

Sterile, reproductive cultures were established by placing a 9 mm^2 plug of culture medium with vegetative mycelium onto the center of each of several oatmeal agar plates. After 3 w, sporulating plates were used to establish additional plates of reproductive fungus on oatmeal agar medium, harvest spores for inoculations, and to identify the fungus as *B*. *cinerea*. Additional reproductive plates were established by inverting a sporulating plate onto a fresh,

oatmeal agar plate and tapping it three times. Harvest of spores was accomplished by flooding sporulating plates with approximately 15 ml of sterile solution of deionized water with 15% glycerol and 0.01% Tween 80, rubbing the fungus for several minutes, and straining the liquid through four layers of sterile cheesecloth. One sporulating plate was submitted to the NCSU Plant Disease and Insect Clinic, which used PCR to verify the identity of the newly isolated rose strain as *B*. *cinerea*.

Each time spores were harvested, solution spore concentration was quantified by counts made under 40x magnification with a Neubauer hemocytometer. The solution, typically between $5 \times 10^6 \cdot ml^{-1}$ and $5 \times 10^7 \cdot ml^{-1}$ spores, was then saved in 40 ml aliquots to serve as inoculum concentrate by freezing at -80 °C. Inoculum was prepared by thawing the frozen inoculum concentrate and diluting it with tap water to yield a final spore concentration of $10^5 \cdot ml^{-1}$. Roses were inoculated by spraying all sides of leaves and flowers with constantly agitated inoculum with a hand-held, household spray bottle to the point of run-off. Total delivered inoculum volume was approximately 40 ml per dozen roses.

Plant material. 'Light Orlando' rose was used because it is currently used in the industry and is known to be susceptible to *B*. *cinerea*. Roses with 45 cm stems were delivered overnight from a commercial grower. Roses to be treated with fungicide and the no-fungicide inoculated control were inoculated with *B*. *cinerea* spores within a couple hours of receipt and then left to incubate wrapped in black plastic bags for 24 h at constant 20 °C. The no-spore control treatments were handled in the same way except flowers were sprayed with spore-free control solutions or left untreated.

Application of treatments. All control and fungicide treatments were imposed after the 24-h incubation period. Most fungicide and control treatments were applied by inverting and dipping rose stems into buckets containing 15 L tap water plus fungicide to within 10 cm of stem bases such that the flower and all foliage was submerged. The treatments took 20 sec: 5 sec to slowly submerge, 5 sec to slowly swirl through solution, 5 sec to raise and drain upside down, 5 sec for 5 downward pulsing shakes. The two essential oil treatments were applied by spraying all sides of leaves and flowers with constantly agitated dilute essential oil solution with a hand-held, household spray bottle to the point of run-off. Total delivered volume was approximately 40 ml per dozen roses. One "fungicide" treatment was the wrapping of the roses in an antimicrobial plastic film (BION plastic wrap, G.CLO USA) rather than swirling flowers in a chemical dip.

Four control treatments were included in the study: 1) "no fungicide control" of spraying roses the same as spore-inoculated flowers at the time of inoculation and tap water submersion at the time of fungicide treatment, 2) "no-spore control" of spraying with the same solution as contained spores (diluted with tap water as the spore-containing inoculum was) but without spores at the time of inoculation and tap water submersion at the time of fungicide treatment, 3) "water only control" of spraying roses with tap water at the time of inoculation and tap water submersion at the time of fungicide treatment, and 4) "absolute control" of not spraying roses at the time of inoculation and no treatment at the time of fungicide treatment, and. All fungicide and control treatments are shown in Table 1.

Following imposition of fungicide and control treatments, roses were again wrapped in black plastic, separated by treatment, and stored at 2 °C for 48 hr. Flowers were then removed

from the bags, cut to 40 cm, and placed in groups of three into jars containing 350 ml tap water. Flowers were held at constant 20 °C under 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light for 12 $h \cdot d^{-1}$ at 40–60% relative humidity (RH).

Assessment of Botrytis damage and phytotoxic responses. Disease development was assessed 1 d after placement into jars and every other day thereafter through 15 d at which time all flowers had been terminated. We used a modification of the decay index of flower petal and receptacle described by Hazendonk (1995) and Meir (1998). The scale is 1 to 8: 1, no symptoms; 2, 1% disease (or 1-4 pinpoint lesions; 3, 2-5% disease (or 5-19 pinpoint lesions); 4, 6-12% disease (or >20 pinpoint lesions; 5, 13-25% disease; 6, 26-50% disease; 7, 51-75% disease; and 8, 76-100% disease (or collapse of flower head at receptacle. Stems were rated as a 0 or 1 for the presence of ≥1 botrytis leaf lesions.

Phytotoxicity responses of flowers and leaves were assessed separately 3 d after placement into jars using a three-point, subjective scale: 0, no apparent damage; 1, slight damage; 3, pronounced damage. In the case of flowers, slight damage was margin damage penetrating <2 mm into petal tissue at any point and pronounced damage was margin damage penetrating ≥2 mm into petal tissue at any point and/or browning of petal creases. Leaves were classified as having slight damage if they were slightly yellowed and/or had darkened margins and as having pronounced damage if they were very chlorotic and/or darkened areas were present on more than just the margins.

Statistical design and analysis. For each run of the experiment a randomized complete block design was used with one jar with three roses for each treatment within each of two blocks and two blocks within each of two repetitions. Two runs of the experiment were carried out such that a total of 24 individual flowers were subjected to each of the 21 treatments. Statistical analyses were conducted with JMP Pro 12 (SAS, Cary, NC). A standard least squares method was used to perform analysis of variance on botrytis damage ratings on flowers and phytotoxicity ratings on flowers and leaves. Fungicide treatment and run were treated as fixed effects, and replication and block were treated as random effects. Binary logistic regression was used to determine the influence of treatments on variables rated as yes/no: presence of botrytis on flowers, presence of botrytis on leaves, and whether the flower was terminated for botrytis symptoms.

Results

Analyses of variance showed that differences among fungicide treatments were highly significant (P<0.001) for all flower and leaf variables assessed on every day observations were made. Phytotoxicity manifest as petal margin necrosis (Fig. 1B) was common, and fungicide treatments differed in the degree to which they caused a phytotoxicity response after 3 d (Table 2). It was clear that the fungicide treatments were responsible for the damage, because no-fungicide control roses did not exhibit similar damage (Table 2).

Fungicide treatments had a highly significant impact (P<0.001) on frequency of flowers and leaves having botrytis and on flower botrytis damage ratings on every day observations were made. The greatest separation among treatments in incidence and severity of botrytis on

flowers and leaves were found on day five and seven, respectively (Table 3). All flower terminations due to botrytis had occurred by 13 d, and influence of fungicide treatment was again found to be highly significant on this variable (P<0.001, Table 3). Botrytis was the cause for flower termination in >50% of the roses in the spore-inoculated, no-fungicide control and 14 of the 17 fungicide treatments (Table 3). Botrytis was the cause for flower termination in 12–25% of individuals even in the non-inoculated control treatments (Table 3). Reduction in frequency of flower terminations due to botrytis compared to control flowers that were inoculated with *B*. *cinerea* spores but treated with water rather than a fungicide after the 24-hr incubation period was 30% or less for 14 of the 17 fungicide treatments (Fig. 1D).

While blocks within replicate and replicates within run of the experiment rarely showed significance for any variable, the whole runs of the experiment differed from each other in several instances (Tables 2 and 3). The second run of the experiment had higher infection rates, more rapidly developing botrytis symptoms, and more severe botrytis damage than the first. For example, frequency of flowers with botrytis after 5 d in the first run was 32% while it was 63% in the second, and flower botrytis damage rating after 5 d was 3.78 for the first run and 4.92 for the second, highly significantly different in both cases (P<0.001). The interaction between fungicide treatment and run was also significant for many of the variables, e.g. flower and leaf phytotoxicity scores after 3 d (Table 2) and flower botrytis frequency and damage rating after 5 d (Table 3).

Discussion

It is not surprising that we observed high rates of botrytis in the 'Light Orlando' cut roses we inoculated. Compared to the typical pathogen pressure, we used a high concentration of *B. cinerea* spores ($10^5 \cdot ml^{-1}$) for inoculations and then provided optimum conditions for spores to germinate and infect plant tissue as described by Sosa-Alvarez et al. (1995) and (Zhang and Sutton (1994) prior to imposing fungicide treatments. The observation of botrytis in the non-inoculated control flowers was also not surprising given that 'Light Orlando' is a rose cultivar known to be susceptible to botrytis and that approximately 10% of flowers evidenced botrytis infection upon receipt.

The large differences in botrytis infection frequency, disease progression rate, and ultimate disease severity between the two runs of the experiment point to the need for future experiments to be repeated in time. This is particularly important given the cases of significant interactions between fungicide treatment and run. In the present experiment, one explanation for differences between runs is the RH in the trial laboratory. Though maintained between 40% and 60%, the first run was conducted while RH was closer to 40% for the duration and the second while RH was closer to 60%. The flowers themselves may have been differentially susceptible to botrytis, because of their production histories or dissimilar shipping and handling conditions at some point.

Phytotoxicity scores for and frequency of botrytis on leaves supported what was observed in flowers. In no case did leaf observations for roses treated with a given fungicide contradict the flower observations for that fungicide. The fact that leaf phytotoxicity was almost always much lower than that for flowers of the same fungicide treatment suggests that it may be possible to streamline future experiments by collecting data from flowers only.

We tested synthetic and non-synthetic products with the aim of revealing new, effective methods for postharvest botrytis control in cut roses. To that end, one synthetic fungicide stood out as having the ideal combination of relatively low phytotoxicity and low botrytis damage in the roses treated with it: fludioxonil (Medallion manufactured by Syngenta) applied at 0.23 g/L. Additionally, treatment with the non-synthetic fungicides cinnamon essential oil and thyme essential oil applied at 0.20% yielded flowers that had relatively low frequency of terminations due to botrytis while exhibiting moderate phytotoxicity.

This work suggests areas for future applied research. Experiments with fludioxonil applied at several concentrations prior to shipping to a large number of cut rose cultivars would elucidate the optimal concentration for minimizing both phytotoxicity and postharvest incidence of botrytis across genotypes. Such work would shed more light on whether there is the potential to use fludioxonil dips to reliably lower postharvest incidence of botrytis in susceptible rose cultivars.

Our findings indicate that additional research with essential oil application to cut roses is warranted because, although essential oils have long been recognized to have effective fungistatic compounds (Wilson et al., 1997), very little is known about utilizing them with cut flowers. Cinnamon and thyme essential oil were among the better treatments for botrytis control in this work, but that benefit came with moderate but unacceptably high phytotoxicity. Testing a range of concentrations and different delivery methods, such as volatilized oil compounds rather than direct spray application, may show that the phytotoxicity can be minimized while significantly reducing incidence of botrytis. This type of work would likely produce useful information for other important cut flower taxa beyond rose cultivars.

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Table 1. Treatments imposed on cut 'Light Orlando' roses after inoculation with of $10^5 \cdot ml^{-1}$ *Botrytis cinerea* spores and incubation in black plastic bags for 24 h at constant 20 °C. Following fungicide treatment roses were stored in black plastic bags for 48 h at constant 2 °C before cutting stems to 40 cm and placing them into jars containing tap water for observation for 15 d. The second column provides treatment reference numbers for Tables 2 and 3 and Fig. 1.

Fungicide treatments	No. in other tables/figures	Label/literature suggested concentration	Amount used per 15 l
Bacillus subtilis (Cease)	1	6 qt/100 gal	227 ml
BASF Exp 703	2	10 fl oz/100 gal	11.8 ml
Batine	3	1 ml/l each of 2 solutions	15 ml each solution
Chlorine bleach (Clorox)	4	400 µl · ml^{-1}	6.0 ml
Chlorothalonil (Daconil)	5	2.5 Tbsp/gal	148 ml
Cinnamon essential oil	6	0.20%	2 drops/50 ml
Copper pentahydrate (Phyton 27)	7	32.5 fl oz/10 gal	38.4 ml
Fenhexamide (Elevate)	8	1.13 lb/100 gal	20.5 mg
FloraDip R	9	4 ml/l	60 ml
Fludioxonil (Medallion)	10	3 oz/100 gal	3.4 g
Hydrogen peroxide (ZeroTol 2.0)	11	30.0 fl oz/ 10 gal	37.5 ml
Iprodione (Chipco 26019 Flo)	12	1 qt/100 gal	37.9 ml
Paraffinic oil (JMS Stylet Oil)	13	2 fl oz/gal	237 ml
Potassium bicarbonate (Milstop)	14	1 Tbsp/gal	45.6 g
BASF Exp 516 (Pageant Intrinsic)	15	15 oz/100 gal	17.0 g
Thyme essential oil	16	0.20%	2 drops/50 ml
Antimicrobial film (BION)	17	Wrap flowers in film, tape closed	0.38 m^2
Control Treatments		Treatment at *B. cinerea* Inoculation	Treatment at Fungicide Application
Spore inoculated but no fungicide	18	Inoculation with *B. cinerea*	Tap water-only dip
No spore	19	Spray with spore solution without spores	Tap water-only dip
Water only	20	Spray with tap water	Tap water-only dip
Absolute	21	Untreated	Untreated

Table 2. Flower and leaf phytotoxicity ratings for 'Light Orlando' cut roses given a fungicide treatment (1 – 17, identified in Table 1) or a non-fungicide control treatment (18 – 21, identified in Table 1). Phytotoxicity response was scored as 0 for none, 1 for moderate, and 3 for severe 3 d after flowers were placed into jars at constant 20 °C with 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light for 12 $h \cdot d^{-1}$ at 40 – 60% relative humidity. Values in a column followed by the same letter are not different at the *P*=0.05 level according to Tukey's HSD.

Treatment no.	Day 3 flower phytotoxicity rating		Day 3 leaf phytotoxicity rating	
1	1.17	ab	0.38	ab
2	0.91	abc	0.34	ab
3	0.42	cde	0.29	ab
4	0.49	cde	0.13	ab
5	0.69	bc	0.12	ab
6	0.74	abc	0.19	ab
7	1.22	a	0.41	ab
8	0.76	abc	0.37	ab
9	0.76	abc	0.25	ab
10	0.16	de	0.00	b
11	0.70	bc	0.33	ab
12	0.53	cd	0.08	b
13	1.18	ab	0.50	a
14	1.24	a	0.51	a
15	0.63	cd	0.29	ab
16	0.78	abc	0.21	ab
17	0.49	cde	0.41	ab
18	0.00	e	0.00	b
19	0.00	e	0.08	b
20	0.00	e	0.00	b
21	0.00	e	0.00	b

Source	ANOVA P-values	
Fungicide	<0.0001	<0.0001
Run	<0.0001	<0.0001
Fungicide x Run	<0.0001	<0.0001
Rep(Run)	0.5986	0.6017
Block(Rep)	0.3088	0.8545

Table 3. Impact of fungicide treatments (1 – 17, identified in Table 1) and a non-fungicide control treatment (18) on 'Light Orlando' cut roses inoculated with $10^5 \cdot ml^{-1}$ *Botrytis cinerea* spores prior to fungicide treatments as measured by frequency of flower`s with botrytis and flower botrytis damage rating (1 = no damage, 8 = worst damage) after 5 d, frequency of leaves with botrytis after 7 d, and frequency of flowers terminated for botrytis after 13 d. "Day" refers to the number of days after flowers were placed into jars at constant 20 °C with 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light for 12 $h \cdot d^{-1}$ at 40 – 60% relative humidity. Control treatments 19 – 21 are identified in Table 1. Values in a column followed by the same letter are not different at the *P*=0.05 level according to Tukey's HSD.

Treatment no.	Day 5 frequency of botrytis on flowers (%)	Day 5 flower botrytis damage rating	Day 7 frequency of botrytis on leaves (%)	Day 13 frequency of flowers terminated for botrytis (%)
1	70.8 abc	5.29 abcd	41.7 abcde	83.3 ab
2	32.0 defg	4.12 bcdef	41.7 abcde	64.0 abcd
3	41.7 def	4.42 bcde	54.2 abcde	75.0 ab
4	72.0 abc	5.40 abc	40.0 abcde	80.0 ab
5	40.0 def	4.32 bcde	44.0 abcde	88.0 a
6	36.0 def	4.36 bcde	44.0 abcde	48.0 bcde
7	76.0 ab	5.60 abc	76.0 a	96.0 a
8	76.0 ab	5.72 ab	60.0 abcd	88.0 a
9	52.0 bcde	4.40 bcde	64.0 abc	64.0 abcd
10	28.0 efg	3.24 efg	16.7 cde	36.0 cde
11	52.0 bcde	4.88 abcd	44.0 abcde	80.0 ab
12	40.0 def	4.00 cdef	32.0 abcde	76.0 ab
13	92.0 a	6.20 a	68.0 ab	100.0 a
14	68.0 abc	5.08 abcd	70.8 ab	92.0 a
15	48.0 cde	4.06 bcdef	52.0 abcde	72.0 abc
16	28.0 efg	3.72 def	28.0 bcde	48.0 bcde
17	76.0 ab	5.56 abc	56.0 abcde	92.0 a
18	56.0 bcd	5.04 abcd	52.0 abcde	92.0 a
19	7.7 g	2.00 g	15.4 de	11.5 e
20	8.3 g	1.83 g	8.3 e	16.7 e
21	16.7 fg	2.54 fg	16.7 cde	25.0 de

Source	ANOVA P-values			
Fungicide	<0.0001	<0.0001	<0.0001	<0.0001
Run	<0.0001	<0.0001	0.0264	0.0166
Fungicide x Run	<0.0001	<0.0001	0.2632	0.1636
Rep(Run)	0.0750	0.1046	0.3909	0.5311
Block(Rep)	0.0848	0.0993	0.1423	0.4580

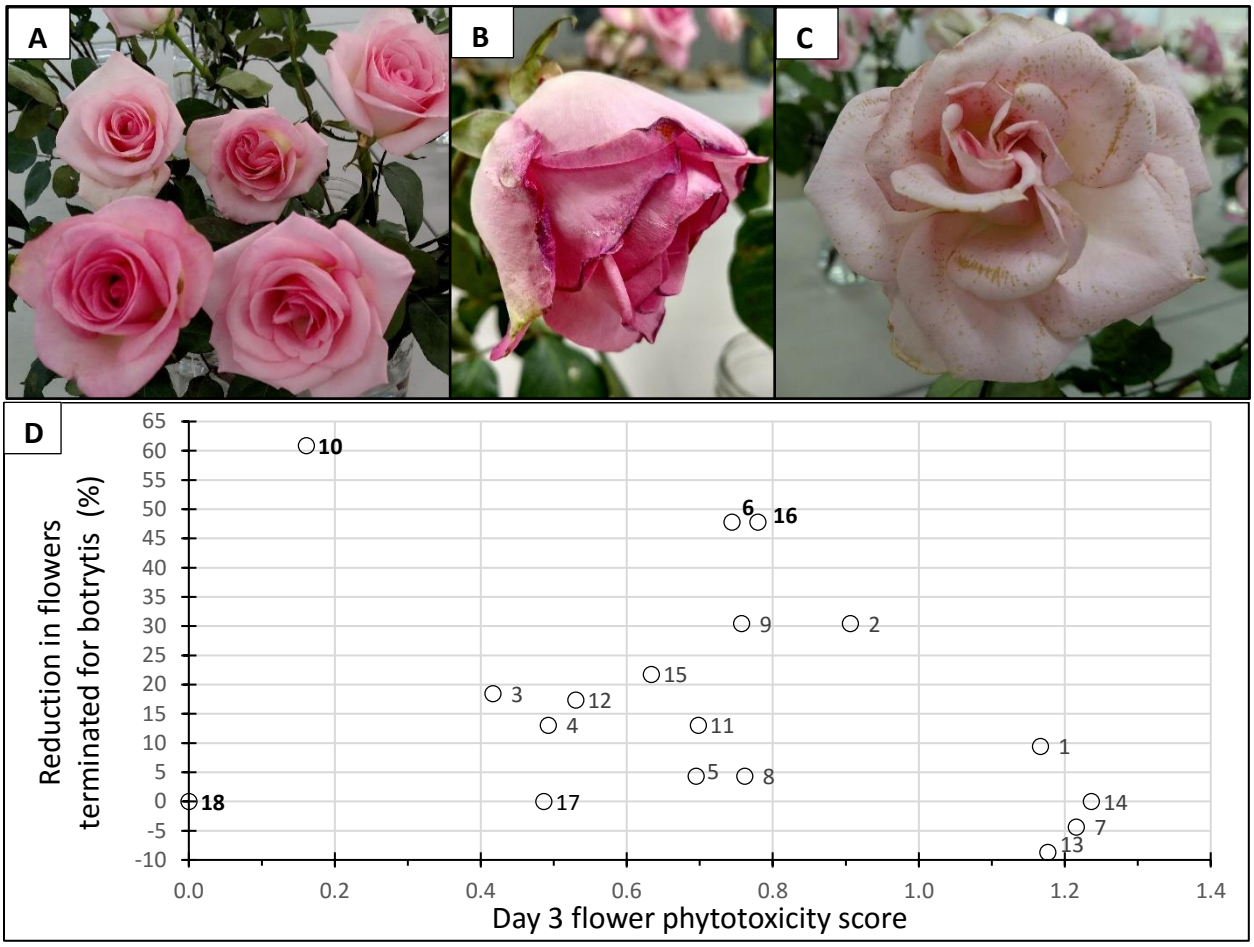

Fig. 1. 'Light Orlando' rose response to fungicide and control treatments: A) "Water-only" control flowers after 3 d, B) Phytotoxicity manifest as petal margin damage after 3 d, C) High level of botrytis infection after 7 d, D) Phytotoxicity score after 3 d and reduction in frequency of flower terminations due to botrytis compared to control flowers (treatment 18) that were inoculated with *Botrytis cinerea* spores but treated with water rather than a fungicide after the 24-hr incubation period. Fungicide treatments 10, 6, and 16 are fludioxonil, cinnamon essential oil, and thyme essential oil, respectively. Other treatments are identified by number in Table 1. Significance of pairwise comparisons among fungicide treatments for phytotoxicity score after 3 d and frequency of flowers terminated due to botrytis are shown in Tables 2 and 3, respectively.